REMARKS

Claims 1-32 represent the currently pending claims. Claims 1-19 and 23-25 have been cancelled. Claims 20-22 and 26-32 currently stand rejected. Claim 20 is amended herein. Upon entry of the present amendments, claims 20-22 and 26-32 constitute the pending claims of the application.

Amendments to the Claims: Applicants have amended claim 20, without prejudice, to more clearly and distinctly point out the subject matter of the claimed invention and to delete the term "preventing" from the scope of the claim. Applicants hereby reserve the right to pursue any deleted subject matter in one or more applications claiming the benefit of priority from the instant application.

Rejection Based on 35 U.S.C. § 112: Claims 20-22 and 26-32 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Specifically, the Office contends that the claims encompass subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicants respectfully traverse this rejection to the extent that it is maintained over the claims as amended herein.

The Office does not dispute that Applicants have demonstrated that the compounds provided in the genus of amended claim 20 are effective in producing a desirable response in the heat-shock experiments of the pending application, as well as in related issued U.S. Patent Nos. 6,653,326 and 7,148,239. As discussed in more detail below, one of ordinary skill in the art at the effective filing date of the instant application would recognize and understand that the heat-shock proteins tested in the experiments disclosed in the application are common molecular chaperones. (*See also*, Original Specification, p. 1, lines 14-19). In response to the alleged lack of evidence connecting the conditions associated with the malfunction of the molecular chaperone system with the disclosed *in vitro* data, Applicants submit herewith Exhibits A-B which demonstrate a nexus between the disclosed *in vitro* data and the claimed conditions.

As mentioned in the first sentence of the Background of the Invention, molecular chaperones are proteins which mediate protein folding. (*See*, Original Specification, p. 1, line 10). Exhibit A (Gething et al., *Nature*, 355, 33-45, (1992)) is one of many publications that characterize heat-shock proteins as being well known molecular chaperones which are involved in the cellular response of organisms to, *inter alia*, heat shock. In addition, Exhibit A illustrates that one of ordinary skill would recognize and understand that the proteins may be induced under a variety of other stress conditions. (*See*, Exhibit A, p. 35, cols. 1-2). Thus, it would be understood by one of ordinary skill in the art that the physiological stress accompanying the condition associated with the molecular chaperone may vary greatly and may come from a wide variety of different physiological stresses, such as those recited in amended claim 20.

Furthermore, as stated in Exhibit B (Welch, *Cell Stress & Chaperones*, 1(2):109-115, (1996)), "protein folding and assembly inside the cell is mediated by a class of proteins now commonly termed molecular chaperones." (*See* Exhibit B, p. 109, col. 1). Exhibit B also states that "[a]bnormalities in protein folding constitute the molecular basis for many human diseases." (*See*, Exhibit A, p. 110, col. 1). Exhibit B, Table 1, entitled "Diseases involving defective protein folding," lists a relatively wide variety of diseases that are known to involve defective protein folding and/or trafficking. Hence, in view of the state of the art as evidenced by Exhibits A and B, the *in vitro* experiments provided in the instant application would have been understood by the skilled worker to demonstrate, at the cellular level, that the subject compounds may be useful for treating conditions associated with the molecular chaperone system, no matter how diverse the physiological stress or associated condition and/or disease.

Rejection Based on 35 U.S.C. § 112: Claim 21 is further rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Specifically, the Office contends that the claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicants respectfully traverse this rejection to the extent that it is maintained over the claims as amended herein.

As discussed above, Exhibits A-B provide the nexus between the *in vitro* experiments described in the instant application and the diseases of amended claim 21. Indeed, Exhibit B not only describes a variety of diseases generally, but points to specific diseases that would be encompassed by pending claim 21. Exhibit B illustrates that cancer (a neoplastic disease) and Creutzfeldt-Jakob disease (an infectious disease caused by abnormal proteins called prions), completely unrelated diseases, nonetheless share an involvement in defective protein folding that may be affected by expression of a molecular chaperone. Accordingly, Applicants submit that claim 21 is also enabled in view of the disclosed *in vitro* experiments and Exhibits A-B.

Rejections Based on Obviousness-Type Double Patenting: Claims 20-22 and 26-32 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 17-34 of U.S. Patent No. 7,148,239 ("the '239 patent). Specifically, the Office contends that both sets of claims refer to treating a disease connected with the function of the chaperone system and that the recited compounds in instant claims 20-22 are obvious variations of the compounds of the methods of the '239 patent. Applicants traverse.

As set forth previously (see page 11 of the February 19, 2008 Amendment and Response), Applicants respectfully submit that the claimed subject matter of the present application and that of the '239 patent were simultaneously filed as divisional applications from the parent application, now the '326 patent, which was subject to a requirement for restriction. As such, the claims of the present application and those of the '239 patent have already been considered to be patentably distinct by the Office, and so the use of the '239 patent as basis for the present rejection is improper. The Office has failed to rebut this point in the outstanding Office Action. For this reason, Applicants request reconsideration and withdrawal of this rejection.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 212.596.9000. Any other fee required for timely consideration of this submission may be charged to Deposit Account No. 06-1075, under Order No. 004049-0015-102 from which the undersigned is authorized to draw.

Dated: December 11, 2008

Respectfully submitted,

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Exhibit A

Protein folding in the cell

Mary-Jane Gething & Joseph Sambrook

In the cell, as *in vitro*, the final conformation of a protein is determined by its amino-acid sequence. But whereas some isolated proteins can be denatured and refolded *in vitro* in the absence of other macromolecular cellular components, folding and assembly of polypeptides *in vivo* involves other proteins, many of which belong to families that have been highly conserved during evolution.

UNTIL recently, scientists using biophysical techniques to study refolding of polypeptides *in vitro* had little need for communication with those investigating the biosynthesis and maturation of proteins within cells. But the realization that the attainment of correct tertiary and quaternary structure is an important determinant of efficient intracellular protein transport[1-3] led to the development of techniques to analyse the early stages of protein folding *in vivo*[4]. These studies have shown that in cells families of abundant proteins modulate and promote protein folding, assembly and disassembly, and facilitate the degradation of malfolded polypeptides. Here we review current knowledge of these proteins and discuss current theories of the mechanisms by which they function.

Protein folding *in vitro* and *in vivo*

Although the three-dimensional structures of several hundred proteins are now known in great detail[5,6], the pathways by which such polypeptides attain their native configurations remain substantially undefined. Anfinsen's classic experiments on the refolding of ribonuclease *in vitro*[7,8] established that all the information required to determine the final conformation of a protein can reside in the polypeptide chain itself: the denatured enzyme can refold into its native conformation in the absence of other proteins. Similar results have since been obtained with several other small, single-domain polypeptides, and with a few larger, more complex proteins (reviewed in refs 9-13). Such studies suggest that refolding *in vitro* may be initiated by (1) collapse of hydrophobic regions into the interior of the molecule, (2) formation of stable secondary structures that provide a framework for subsequent folding, and (3) formation of covalent interactions, such as disulphide bonds, that stabilize the polypeptide in particular conformations. Evidence has been obtained in support of each of these mechanisms and it is likely that all three may operate in conjunction during the early stages of refolding. Subsequent folding seems to occur through a limited number of pathways involving distinct intermediates ('molten globules'[14,15] or 'compact intermediates'[13]) that have significant secondary structure and a compact form but lack a well-defined tertiary structure and expose more hydrophobic surface than fully folded molecules. These intermediates seem to be in rapid equilibrium with the fully denatured state and are only slowly converted to the native state. Thus the rate-limiting step in the refolding process frequently occurs at a very late stage, just before the protein adopts its final, native conformation.

Despite their value in defining the types of intramolecular interactions that drive polypeptide folding, *in vitro* experiments do not accurately reflect the process of folding of nascent proteins in the interior of a cell. Refolding *in vitro* is frequently very inefficient in comparison to folding *in vivo*, and often requires protein concentrations and physicochemical conditions very different from these occurring intracellularly. Furthermore, although refolding experiments involve the whole polypeptide chain, the opportunity exists *in vivo* for folding to commence as soon as the N-terminal portion of the nascent chain emerges from the ribosome (or from the lipid bilayer following membrane translocation). Finally, many proteins exist in cells as homo- or hetero-oligomeric complexes that in some cases are assembled before folding of the individual polypeptide chains is complete. The probability is slight that such complexes could form *in vitro* at the low subunit concentrations occurring intracellularly[16]; the probability is even lower that they could form before individual chains have become irreversibly malfolded.

To investigate protein folding *in vivo* it was first necessary to devise assays for polypeptide conformation that do not depend on obtaining large quantities of partially folded proteins that are sufficiently pure for physicochemical measurements. The first such assays analysed the formation of disulphide bonds during the folding of biosynthetically labelled secretory proteins and showed that these bonds can form even before synthesis of a polypeptide is completed, and that disulphide bond formation occurs *in vivo* at a significantly faster rate than can be achieved under the most favourable conditions *in vitro*[17]. Subsequently, additional *in vivo* folding assays were developed that use conformation-specific antibodies, protease sensitivity, or sucrose density gradient centrifugation to probe the tertiary and quaternary structure of radiolabelled proteins (reviewed in ref. 4). Studies using these techniques showed that although individual domains of a nascent polypeptide may fold very rapidly, acquisition of the final native structure of the whole protein can proceed comparatively slowly. Furthermore, partially folded intermediates whose structures would seem unlikely to be stable in the presumably aqueous environment of the cell's interior can lie dormant for many minutes to many hours before folding is completed[3,18,19]. Despite such extended pauses in the assembly process, folding *in vivo* of wild-type proteins is usually highly efficient with > 95% of the newly synthesized polypeptides eventually attaining their native three-dimensional structures[3,20]. Polypeptide misfolding and aggregation, frequently a major problem during refolding *in vitro*[21], rarely occurs *in vivo* except with mutant proteins or during synthesis at elevated temperatures. Finally, partially folded polypeptides can frequently be isolated as complexes with specific cellular proteins, notably members of stress protein families[5,22-26].

The rest of this article summarizes the results of studies that have revealed the existence of at least two classes of proteins involved in polypeptide folding in cells. The first class includes conventional enzymes that catalyse specific isomerization steps that may otherwise limit the rate of folding of some proteins, whereas a second class of 'chaperones' stabilize unfolded or partially folded structures and prevent the formation of inappropriate intra- or interchain interactions. Some members of this second class also interact with apparently native protein molecules to promote rearrangement of protein–protein interactions in oligomeric structures.

Enzymes involved in protein folding

In vitro, two rate-determining steps involving isomerization of covalent bonds can be catalysed by purified cellular enzymes. Protein disulphide isomerase (PDI) catalyses thiol/disulphide interchange reactions and, depending on the nature of the polypeptide substrate and the imposed redox potential, promotes protein disulphide formation, isomerization or reduction[27,28]. PDI does not determine the polypeptide's folding pathway, but rather facilitates formation of the correct set of disulphide bonds

by promoting rapid reshuffling of incorrect disulphide pairings. Proteins with peptidyl prolyl *cis–trans* isomerase (PPIase) activity catalyse the otherwise slow isomerization of X–P peptide bonds (where X is any amino acid and P is proline in single-letter amino-acid code) and can accelerate the refolding of proline-containing polypeptides *in vitro*[10,29,30] and *in vivo*[31].

PDI and the thioredoxin-like proteins

Several lines of evidence suggest that PDI activity is required for folding of nascent polypeptides in the endoplasmic reticulum (ER) of eukaryotic cells. Thus the abundance of the enzyme in the ER of different cell types correlates with the level of secretory protein synthesis[17] and *in vivo* chemical crosslinking studies demonstrate a specific association between the enzyme and newly synthesized immunoglobulin chains in the ER (ref. 32). Finally, reintroduction of purified PDI into microsomes evacuated of their lumenal content by alkali or detergent treatment restores cotranslational formation of disulphide bonds in proteins synthesized using a cell-free system[33]. PDI is an essential protein in yeast[34,35], but the biological process whose disruption leads to lethality has not yet been defined.

Mammalian PDI is a dimer of identical subunits of relative molecular mass 57,000 (M_r 57K), each of which contains duplications of domains showing strong homology to thioredoxin[36], a small redox protein present in all classes of organisms from bacteria to higher eukaryotes[37]. Computer modelling studies based on the known three-dimensional structure of *Escherichia coli* thioredoxin indicate that a functional PDI dimer contains four thioredoxin-like domains each having a dithiol/disulphide active site located on a prominent loop at the surface of the molecule[38].

PDI's role in the ER may not be limited to disulphide isomerization[28]. First, many types of mammalian cells contain, in addition to substantial amounts of homodimeric PDI, the enzyme prolyl-4-hydroxylase which causes extensive modification of proline residues in nascent collagen molecules. This enzyme, an $\alpha_2\beta_2$ tetramer whose β-chain dimers are identical to PDI, also has PDI activity (reviewed in ref. 27). The 64K α-chains form the binding site for the peptide substrate to be hydroxylated, but it has not yet been determined whether the dithiol/disulphide active sites of the PDI/β-subunits are directly involved in the hydroxylation reaction. Second, the 'glycosylation site binding protein' (GSBP) component identified using a glycosylation site photoaffinity probe[39] is identical to PDI (ref. 34). However, involvement of this enzyme during *N*-linked glycosylation in the ER is not proved, as depletion of PDI from microsomes does not affect their capacity to support oligosaccharide addition to nascent polypeptides[40]. Finally, PDI has also been identified as a component of the microsomal triglyceride transfer protein complex[41].

Recent studies have identified other ER proteins containing thioredoxin homology units. ERp59, ERp61 and ERp72, three members of a set of proteins whose synthesis is induced at onset of immunoglobulin secretion in murine B cells[42], have been characterized by complementary DNA cloning and sequencing. ERp59 is identical to PDI (ref. 43). Although ERp61 contains two thioredoxin-like domains found in the same relative positions as in the PDI molecule, the rest of its sequence is unrelated to that of PDI (R. Mazzarella and M. Green, personal communication). ERp72 contains three thioredoxin homology units, two of which are spaced as in PDI, embedded in otherwise unrelated sequences[43]. Neither ERp61 nor ERp72 have PDI activity *in vitro*. Finally, an essential gene, *EUG1*, encoding another PDI-related ER protein containing thioredoxin homology units but lacking PDI activity, has been identified in *Saccharomyces cerevisiae* (C. Tachibana and T. Stevens, personal communication). Whether the unknown functions of these proteins use the redox activity of their thioredoxin-related structural units is still in question. In this context it is of interest that thioredoxin itself is required for assembly of filamentous phages in *E. coli*, playing a part that does not involve its redox activity as site-specific mutation of either or both of the active site cysteines does not alter the ability of the mutant proteins to support phage assembly (for review, see ref. 44). Thioredoxin is thought to confer processivity on the reaction that leads to the displacement of the intracellular phage protein pV from single-stranded phage DNA and to its replacement at the membrane by the major coat protein, pVIII.

Thus the ER houses an extended family of enzymes (Table 1) that may use thioredoxin-like domains containing dithiol/disulphide active sites to carry out various functions in the co- and post-translational modification of secretory proteins. Whether these proteins may also have roles in protein assembly that do not involve redox activity, and whether there may be, in addition to thioredoxin itself, other members of this family located in other compartments of the cell remains to be determined.

Peptidyl prolyl *cis–trans* isomerases

Proteins with PPIase activity are highly abundant and widely distributed, being found in virtually all tissues and organisms, from bacteria to mammals (reviewed in ref. 10). Those proteins characterized so far fall into two structurally unrelated families (Table 1), which are named after the clinically important immunosuppressive agents that inhibit their isomerase activity. Thus the eukaryotic cyclophilin proteins bind cyclosporin A (CsA) with high affinity, whereas the FK506-binding proteins bind the structurally distinct compounds FK506 and rapamycin (reviewed in ref. 45). Both CsA and FK506 mediate their immunosuppressive action by preventing the transcription of genes involved in activation of T lymphocytes, whereas rapamycin potently inhibits the response of T cells to the lymphokine IL-2 (ref. 45). These immunosuppressive drugs do not act through inhibition of the PPIase activity of T cells as they are effective at concentrations far below those of the PPIase enzymes, and they inhibit distinct signalling pathways. Rather it seems that cyclophilin and FKBP bind the drugs, which are cyclic peptides, and present them (to as yet undefined targets) in a bioactive conformation that may require a *cis–trans* isomerization around one of their peptide bonds[46,47].

Although most of the PPIase activity in cells is found in the cytosol, family members are located in different cellular compartments. Thus cyclophilin-like proteins are also present in the periplasmic space of *E. coli* cells[48,49], and in the mitochondrial matrix of *Neurospora crassa*[50]. Furthermore, the nucleotide sequences of cyclophilin-related genes cloned from *S. cerevisiae*[51], *Drosophila melanogaster*[52,53] and vertebrate cells[54–57] and of the FKBP-related gene from human cells[58] each encode a stretch of N-terminal amino acids whose sequences are compatible with function as signal peptides for translocation into the lumen of ER. Following their translocation into the ER, some cyclophilin-related proteins may also be transported along the exocytic pathway and secreted into the extracellular medium[55,56]. Consistent with this diversity of localization are the findings that multiple genes encoding cyclophilin- and FKBP-related proteins are present in mammalian cells and lower eukaryotes[58–61], although in *N. crassa* a single gene encodes both cytosolic and mitochondrial cyclophilins[50]. Genetic studies in lower eukaryotes demonstrated that the cyclophilin and FKBP gene products that mediate sensitivity to CsA or FK506 and rapamycin are not essential for cell viability either individually[61–63] or in combination[63], either because of the presence in cells of additional proteins having PPIase activity or because this activity is not required for cell survival.

Despite our partial understanding of their adventitious role in the suppression of T-lymphocyte function[45], the real *in vivo* role and physiological substrates of enzymes with PPIase activity remain to be established. It seems likely that these highly abundant and widely distributed proteins normally act as 'conformases' (ref. 10) catalysing slow steps in the initial folding and/or rearrangement of protein structures. Initial evidence in support

TABLE 1 Enzymes and chaperones that may be involved in protein folding and assembly in cells

Organism/organelle	Enzymes			Chaperones		
Protein family	PDI	Cyclophilin PPIase	FKBP PPIase	Hsp60 (Chaperonin-60)	Hsp70 (Stress-70)	Hsp90 (Stress-90)
E. coli						
Cytosol	Thioredoxin	PPIase b		GroEL	DnaK	HtpG (C62.5)
Periplasm		PPIase a (Rotamase)				
Yeast						
Cytosol		Cph1p (Cpr1p)	Fkb1p (Fkr1p) (Rbp1p)		Ssa1–4p	Hsp83 Hsc83
ER	PDI Eug1p	yCyPB			Kar2p (BiP)	
Mitochondria				Hsp60 (Mif4p)	Ssc1p	
Drosophila						
Cytosol		CyP			Hsp68 Hsp70 Hsc1,2,4	Hsp83
ER	PDI	NinaA				
Mammals						
Cytosol		Cyclophilin (PPIase) (CyPA)	FKBP		Hsp70 (p73) Hsc70 (p72) (CUATPase) (Prp73)	Hsp90 (Hsp83) (Hsp87)
ER	PDI (ERp59) GSBP ERp72 ERp61	CyPB(rCyPLP)			BiP (Grp78)	Grp94 (ERp99) (endoplasmin)
Mitochondria				Hsp60 (Hsp58)	Hsp70 (Grp75)	
Plants						
Cytosol						
ER	PDI				b70 (BiP)	
Chloroplasts				RuSBP		

Six protein families have been identified whose members include enzymes or chaperones proposed to be involved in folding, assembly, rearrangement or degradation of proteins in cells. Members that have been characterized to date from a variety of different organisms are shown. Alternative names are shown in parentheses. For references see the text.

of this hypothesis came from the discovery that the *D. melanogaster ninaA* gene product, an eye-specific cyclophilin-related membrane protein, is required for the folding and/or stability of rhodopsins 1 and 2 (refs 52, 53, 64). Treatment of chick embryo fibroblasts with cyclosporin A delays the folding of the triple helix of type I collagen, indicating a physiological role for cyclophilin PPIase in folding in the ER (ref. 31).

The mechanism by which the two classes of PPIases catalyse rotation around specific peptide bonds also remains to be determined. Human cyclophilin and FKBP display dramatic differences in substrate specificity. Although cyclophilin has a broad specificity and does not discriminate between P_1 amino-acid residues, FKBP has a narrow specificity with a preference for hydrophobic residues at the P_1 position[65]. The reason for this preference became clear with the determination of the three-dimensional structure of FKBP (refs 47, 66), as the folding topology provides a large cavity, lined with conserved aromatic residues, that serves as the active site and drug binding pocket. Mechanistic studies (reviewed in ref. 45) suggest that both cyclophilin and FKBP catalyse the interconversion of *cis*- and *trans*-rotamers of peptide substrates by noncovalent stabilization of a twisted amide transition state.

Protein chaperones

A number of other cellular proteins, now collectively known as chaperones[67], function *in vivo* not as catalysts of secondary structure formation, but rather to recognize and stabilize partially folded intermediates during polypeptide folding, assembly and disassembly. The majority of the currently identified chaperones belong to three highly conserved protein families, whose members are widely distributed from prokaryotes to plants and mammals (Table 1). In eukaryotic cells, different family members are found in different cellular compartments and organelles. As their names imply, proteins of the hsp70, hsp90 and chaperonin (groEL/hsp60) families first came to attention because of their specific induction during the cellular response of all organisms to heat shock (reviewed in refs 68–71). Nevertheless, the majority of the family members are expressed constitutively and abundantly in the absence of any stress, and genetic studies show that many of these proteins are essential for cell viability under normal conditions of growth[68,72,73]. Many hsp family members, including those that do not respond significantly to heat shock, are induced under a variety of other stress conditions[68,74,75] whose common denominator may be the accumulation of unfolded or malfolded proteins in cells[76–79]. To reduce confusion arising from the use of the term 'hsp' to describe all family members regardless of their response to heat shock, we will use the terms 'stress-70' and 'stress-90' for members of the 70K and 90K families, respectively, a nomenclature similar to that suggested by Craig[68,80] for the 70K family of *S. cerevisiae*. The 60K (groEL/hsp60) family is currently described by the term 'chaperonin-60'[81,82].

Chaperones seem to serve many functions that stem from their ability to recognize and modulate the state of folding of polypeptides within cells (Table 2). Thus members of the stress-70 family have been implicated in the stabilization or generation of unfolded protein precursors before assembly in the cytosol[83] or translocation into organelles including the ER and mitochondria[84–86], in stabilization of newly translocated polypeptides before folding and assembly[3,23,87–90], in rearrangement of protein oligomers[72,91,92], in dissolution of protein aggregates[87,93], and in the degradation of rapidly turned-over

cytosolic proteins[94,95]. Stress-90 proteins seem to stabilize a variety of target proteins in an inactive or unassembled state[68]. Chaperonin-60 proteins bind unfolded precursors before export of secretory proteins[24,26] or assembly of protein oligomers[96] in *E. coli*, and help in folding and assembly of polypeptides translocated into chloroplasts or mitochondria in eukaryotic cells[97–99]. In addition, unrelated cellular proteins that are not members of any of these stress protein families have been implicated in protein in cells. Nucleoplasmin, for which Laskey[100,101] coined the term chaperone in 1978, binds to histones and facilitates ordered nucleosome assembly in the nucleus. In the ER, the T-cell receptor-associated protein, TRAP (ref. 102) or p28 (ref. 103), is noncovalently associated with the newly synthesized CD3 chains until they assemble with other subunits of the receptor, whereas an 88K protein transiently associates with newly synthesized major histocompatability complex (MHC) class I heavy chains[104]. In *E. coli*, SecB and trigger factor also bind unfolded secretory precursors before their export across the plasma membrane[26,105–107], PapD has been proposed to act as a chaperone in the periplasmic space to enhance the folding and assembly of components of P pili[108,109], and 'scaffolding proteins' encoded by bacteriophages promote the assembly of phage coats although they are not finally incorporated into the viral particles[110]. With the realization that polypeptide folding in the cell frequently requires the assistance of chaperones, some with a broad target specificity and some dedicated to assembly of particular macromolecules, it seems inevitable that many more such proteins will be identified in the coming months and years.

The stress-70 protein family

The role of stress-70 proteins during the heat-shock response has been studied extensively for many years (for recent reviews see refs 68-71), but only recently has their importance in normal cellular processes such as protein folding, assembly, disassembly and degradation (Fig. 1) become widely appreciated[87,95,111,112]. Stress-70 family members implicated in such processes include in *E. coli*, DnaK; in yeast the cytosolic proteins Ssa1p and Ssa2p, the ER protein Kar2p and the mitochondrial protein Ssc1p; and in mammalian cells the cytosolic proteins hsp70 (p72), hsc70 (p73, clathrin uncoating ATPase) and prp73 (peptide recognition protein 73), and the ER protein BiP (also known as grp78). Although in no case is the interaction between an individual stress-70 protein and its target polypeptide understood in molecular detail, the available evidence reveals several common features that point to a conserved mechanism for the action of these ubiquitous proteins. These features include (1) differential recognition of target polypeptides and modulation of their conformation or state of assembly, (2) involvement of ATP binding and/or hydrolysis, (3) a requirement for other heat shock proteins or cellular factors, and (4) the induction of synthesis of individual stress-70 family members by the accumulation of unfolded proteins in the appropriate cellular compartment.

***E. coli* DnaK.** DnaK, originally, defined as the product of a host gene required for bacteriophage λ DNA replication in *E. coli*, also plays fundamental roles in normal cellular physiology[72]. Mutations in the *dnaK* gene result in temperature-sensitive growth of *E. coli*, overproduction of other heat-shock proteins even at permissive temperatures, impaired synthesis of DNA and RNA and a generalized defect in proteolysis. Furthermore, the synthesis of DnaK is increased as a result of the accumulation in cells of unfolded polypeptides[113] and DnaK binds foreign eukaryotic proteins expressed in *E. coli*[114]. Finally, overproduction of DnaK aids the export of lacZ hybrid proteins across the bacterial inner membrane[115]. These observations all indicate the involvement of DnaK in modulating many protein–protein interactions *in vivo*. Detailed *in vitro* studies have now illustrated the ability of DnaK to interact with either fully assembled or unfolded polypeptide substrates. During bacteriophage replication, DnaK functions together with two other heat shock proteins, DnaJ and GrpE, to release lambda P protein from the inactive pre-primosomal replication complex[72,116,117]. DnaK also functions together with DnaJ to activate RepA initiator protein for binding to the origin of replication of plasmid P1 (ref. 118). Hydrolysis of ATP, thought to be catalysed by DnaK, is required during both reactions, and *in vitro* this ATPase activity of DnaK can be stimulated up to 50-fold in the simultaneous presence of DnaJ and GrpE (ref. 119). Finally, DnaK, which often associates with *E. coli* RNA polymerase through many purification steps, can protect the enzyme from heat inactivation *in vitro*[93]. ATP is not required for the protective effect. DnaK can also reactivate heat-inactivated RNA polymerase by dissolving aggregates formed at high temperatures but this process is absolutely dependent on the hydrolysis of ATP.

Cytosolic stress-70 proteins of eukaryotes. On heat shock of mammalian cells, both the constitutively expressed hsc70 proteins and the heat-induced hsp70 proteins migrate from the cytoplasm to the nucleus where they associate with polypeptides that form an insoluble complex at the increased temperature (reviewed in ref. 111). Subsequently the stress-70 proteins also migrate to the nucleolus and associate with partially assembled proribosomes. Presumably, at elevated temperatures nuclear proteins become partially denatured, exposing hydrophobic regions that tend to interact to form insoluble aggregates. Pelham[87,120] proposed that by binding to the exposed hydrophobic surfaces, stress-70 proteins could limit such interactions and perhaps promote disaggregation. In cell extracts, the stress-70 proteins could be released from their association with nucleolar proteins by addition of ATP, but not nonhydrolysable ATP analogues[120].

In *S. cerevisiae*, the constitutively expressed cytosolic hsc70 proteins Ssa1p and Ssa2p are required, together with a separate

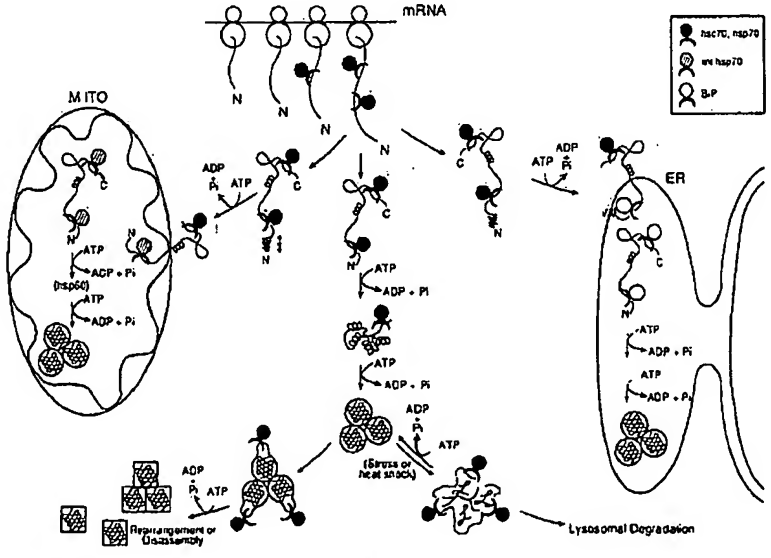

FIG. 1 Illustration of the proposed roles of stress-70 proteins in eukaryotic cells during the folding and membrane translocation of nascent polypeptides, during molecular rearrangements or disassembly, in protection from stress and in protein turnover.

TABLE 2 *In vivo* roles of protein chaperones

	Target polypeptide	Chaperone	Role	Reference
E. coli	Secretory precursors (β-lactamase, proOmpA, prePhoE)	GroEL SecB Trigger Factor DnaK?	Antifolding before translocation	24, 26, 105, 106, 107, 115, 194
	DNA replication complexes	DnaK/DnaJ	Rearrangement of protein complex	72, 116, 117
	Bacteriophage head or tail proteins	GroEL/GroES Bacteriophage scaffolding proteins	Head or tail assembly	96 110
	Foreign proteins	DnaK	Stabilization of unfolded protein?	114
	P pili in periplasmic space	PapD	Pili assembly	118
Photosynthetic bacteria	Rubisco	GroEL/GroES	Oligomer assembly	82, 188, 192, 195, 196
Chloroplasts	Rubisco	RuSBP	Oligomer assembly	81, 191, 192
Mitochondria	Mitochondrial precursors	Hsp70	Completion of translocation Stabilization of prefolded structures in matrix	90, 147 90
	Precursors in matrix	Hsp60	Stabilization of prefolded structures and folding Re-export of precursors to intermembrane space	204–206 204
ER	Nascent secretory proteins	BiP	Completion of translocation Stabilization of prefolded structures in lumen	138 3, 23, 89, 133, 134
	Mutant or foreign proteins	BiP	Stabilization of unfolded structures?	3, 151, 152
	Subunits of T cell receptor MHC Class I heavy chains	TRAP or p28 p88	Receptor assembly Stabilization of newly synthesized heavy chains?	102, 103 104
	Plant storage proteins	b70 (BiP)	Stabilization of newly synthesized polypeptides?	127, 132
Cytosol	Nascent polypeptides	Hsc70 (Hsp70?)	Stabilization of prefolded structures?	83
	Mitochondrial and secretory precursors	Hsc70 (Hsp70?)	Antifolding before translocation	84–86
	Clathrin-coated vesicles	Hsc70 (clathrin uncoating ATPase)	Binds exposed loop of clathrin light chain to promote uncoating	91, 92
	Aged? proteins	Hsc70 (Prp73)	Targeting to lysosomes for degradation	94, 95, 124
	Steroid receptors	Hsp90	Stabilizes inactive form of receptor	178–180
	Retroviral transforming proteins	Hsp90	Stabilizes inactive form of protein during transit to plasma membrane	176, 177
	Actin, tubulin	Hsp90	Stabilizes protein subunits?	68
Nucleus	Preribosomes	Hsp70/Hsc70	Protection of heat denatured proteins	111
	Histones	Nucleoplasmin	Nucleosome assembly	100, 101

N-ethylmaleimide(NEM)-sensitive cytosolic factor, for membrane translocation of secretory and mitochondrial precursors[84–86]. Consistent with this role is that the synthesis of cytosolic stress-70 proteins is induced in yeast cells by the accumulation of secretory precursors in the cytoplasm[79]. Because in cell-free experiments the need for the stress-70 proteins can be eliminated by urea-mediated unfolding of precursors[84,86], the hsc70 proteins are though to promote a translocation competent state by relaxing the tertiary structure of the polypeptides or by dissolving aggregates of untranslocated precursors[84,85]. *In vivo*, the hsc70 proteins may bind to nascent secretory precursors before they fold, maintaining them in a translocation-competent state before membrane penetration. Both Ssa1p and Ssa2p bind ATP with high affinity[84,85], but the involvement of ATP during their interaction with secretory proteins has not been established. *In vitro* experiments indicate that mammalian hsc70 can function in the same manner as Ssa1p and Ssa2p to stimulate import of M13 procoat into mammalian ER microsomes[121]. Hsc70 also interacts transiently with newly synthesized cytosolic proteins[83], presumably to facilitate their proper folding and subsequent assembly in the cytoplasm.

In vivo the targets of the cytosolic stress-70 proteins are not limited to damaged proteins or nascent polypeptides as hsc70, in the form of clathrin-uncoating ATPase[122,123] also promotes the disassembly of clathrin cages by displacing triskelions from the clathrin lattice in a process that requires ATP hydrolysis and the presence of clathrin light chains (reviewed in ref. 91). Transient changes in Ca^{2+} and/or K^+ concentrations in the cytosol surrounding a newly invaginated clathrin-coated vesicle apparently cause exposure of a stretch of amino acids (residues 47–71) in LC_a light chains that comprise a binding site for hsc70 (ref. 92). The interaction between the LC_a peptide and hsc70,

which *in vitro* both alters the conformation of the hsc70 molecule and stimulates its ATPase activity, then initiates the uncoating process which proceeds in a cooperative manner. That the hsc70 binding site on the LC_a molecule is cryptic under the ionic conditions normally present in the cytosol explains why the large cellular excess of hsc70 over clathrin does not lead to a permanent state of clathrin disassembly[92].

Finally, prp73, a stress-70 family member that is almost certainly identical to hsc70, is involved in the lysosomal degradation of intracellular proteins[94]. Prp73 binds peptide sequences (KFERQ and related sequences[95,124]) that target intracellular proteins for lysosomal degradation in response to serum withdrawal. When lysosomal uptake and degradation of protein substrates is reconstituted *in vitro*, prp73 stimulates degradation in an ATP-dependent manner[94]. Serum starvation causes induction of prp73, which presumably alters the conformation of KFERQ-containing proteins so that they can be translocated across the lysosomal membrane.

Stress-70 in the endoplasmic reticulum. The ER of eukaryotic cells contains a roughly 78K member of the stress-70 protein family[79,125-127], now named BiP[23,88]. In mammalian cells, this protein was originally described independently as the immunoglobulin heavy chain binding protein[22,128], and the glucose-regulated protein, grp78 (ref. 129). In yeast cells, BiP is the product of the *KAR2* gene[79,126], one of a class of genes involved in nuclear fusion following mating of yeast cells[130,131]. Under normal growth conditions, BiP is synthesized constitutively and abundantly and comprises about 5% of the lumenal content of the ER of mammalian cells. Its synthesis can be further induced by the accumulation of secretory precursors[79] or mutant proteins in the ER (refs 78, 132), or by a number of different stress conditions[75] that also lead to the accumulation in the ER of unfolded polypeptides[78]. BiP associates transiently with a variety of nascent wild-type exocytotic proteins[3,23,89,133,134] and more permanently with malfolded or unassembled proteins whose transport from the ER is blocked[3,23,135]. Complexes between BiP and nascent secretory proteins, isolated from extracts of mammalian cells, can be dissociated *in vitro* by the addition of ATP, but not of nonhydrolyzable analogues or ADP (ref. 125). BiP, an essential protein in yeast[79,126,136], is therefore thought to have a role in the folding and assembly of newly synthesized proteins in the ER lumen[3,23,87,88]. Initial suggestions that BiP might recognize and retain unfolded proteins in the ER[3,23,87,137], or target malfolded proteins for destruction[135] are no longer tenable. Thus, although unassembled immunoglobulin heavy chains are secreted if the BiP-binding domain is deleted[137], a truncated form of influenza haemagglutinin that does not bind BiP is neither secreted, nor degraded more slowly, than other transport-defective haemagglutinin mutants that do bind to BiP (M. Segal, J.F.S. and M.-J.G., unpublished results). In addition to modulating protein folding in the ER lumen, BiP may be directly or indirectly involved in translocation of precursors across the ER membrane. Yeast cells expressing a temperature-sensitive *kar2* mutant accumulate secretory precursors in the cytosol at the nonpermissive temperature[138]. In addition, *KAR2* interacts genetically with *SEC63* (J. Vogel and M. Rose, personal communication), a yeast gene that encodes a transmembrane protein required for ER translocation of secretory precursors[139]. Sec63p spans the ER membrane and extends into the lumen a domain containing sequences homologous to DnaJ (ref. 140), another heat-shock protein frequently required for DnaK's function in *E. coli*[72,118,119]. Whether cooperation with DnaJ-related proteins is required for the chaperone activity of all stress-70 family members remains to be determined.

Stress-70 in the mitochondrial matrix. Stress-70 proteins have been identified in the mitochondria of a number of organisms including *S. cerevisiae*[141], *Euglena gracilis*[142], *Trypanosoma cruzei*[143] and mammals[144,145]. The yeast protein (Ssc1p) has been localized to the mitochondrial matrix[90] where it performs an essential function, as disruption of the *SSC1* gene is lethal[146]. The amino-acid sequence of Ssc1p is more closely related to DnaK than are those of the other eukaryotic stress-70 proteins[141], consistent with the presumed endosymbiotic origin of mitochondria. Studies using a temperature-sensitive *ssc1* yeast mutant[90] demonstrate dual functions for Ssc1p that parallel those suggested for the ER-located BiP protein. These are involvement in translocation of precursor proteins through the lipid bilayer at mitochondrial contact sites, and involvement in folding of the imported polypeptides in the mitochondrial matrix. The translocation defect can be circumvented *in vitro* by artificially denaturing the precursor molecules, allowing investigation of events in the matrix of isolated mitochondria containing mutant forms of Ssc1p. Interestingly, the imported precursors remain in an unfolded state and can be isolated in physical association with the mutant Ssc1p protein. On the basis of these observations, Kang *et al.*[90] proposed that Ssc1p binds the precursor polypeptide as it emerges on the matrix side of the translocation apparatus in contact sites[97], supporting the continuation of translocation by 'pulling' the precursor into the matrix space. Subsequently, Ssc1p would maintain the imported precursor in an unfolded state until it is released, possibly in an ATP-dependent step, for subsequent folding catalysed by other components in the mitochondrial matrix (see Fig. 2 and discussion below of the role of the chaperonin hsp60). The isolation by crosslinking of complexes containing a partially translocated precursor, a mitochondrial outer membrane protein (ISP42) and Ssc1p provides direct evidence that Ssc1p can interact with polypeptides before the chains have been completely imported into the mitochondrial matrix[147].

Role of ATP binding and hydrolysis by stress-70 proteins. All stress-70 family members bind ATP and a number of them, including DnaK (ref. 16), hsc70 (ref. 122) and BiP (ref. 148), have weak ATPase activities that can be elicited by appropriate protein substrates and by some but not all synthetic peptides[92,148]. Adenine nucleotide binding apparently causes conformational changes in stress-70 proteins that result in altered sensitivity to proteases[149], or in alteration of their oligomeric state[150]. ATP and ADP differ in their effects. ATP protects a roughly 60K fragment of BiP while ADP protects a roughly 45K fragment; ATP stabilizes the monomeric form of hsc70 whereas ADP stabilizes the dimer. These consequences of ATP binding do not require hydrolysis as the nonhydrolysable analogue ATPγS can substitute. By contrast, addition of ATP, but not of nonhydrolysable analogues, to cell extracts causes dissolution of complexes between stress-70 proteins and their polypeptide substrates, including DnaK and bacteriophage λP protein[117], hsp70 and heat-shocked nuclei[120], hsc70 and mutant forms of the cellular p53 protein[114], and BiP and immunoglobulin heavy chains[125]. These observations led Pelham[87,120] to propose that ATP hydrolysis causes conformational changes in stress-70 proteins that are transmitted to the substrates, promoting their folding or weakening their interactions with other polypeptides. However, it is currently believed that binding of stress 70 proteins may simply stabilize unfolded conformations of their target polypeptides, preventing the formation of inappropriate intra- or intermolecular interactions. Rothman[16] has proposed that ATP hydrolysis, which takes place *in vitro* with a turnover time of about 5 min, provides a timed mechanism of release of the stress-70 protein from its substrate, freeing the polypeptide to continue the folding process. This turnover time could of course be altered *in vivo* by interactions with other cellular components. As discussed earlier, the ATPase activity of DnaK can be stimulated 50-fold in the presence of DnaJ and GrpE proteins[119].

Substrate recognition by stress-70 proteins. The molecular basis of substrate recognition by stress-70 proteins remains a matter for speculation. Prp73 binds cytosolic proteins that contain sequences identical or closely related to the consensus pentapeptide KFERQ[94,95,124]. Other stress-70 family members interact with a variety of polypeptides that do not contain any conserved

motif, suggesting that their 'recognition signals' do not consist of unique linear amino-acid sequences. Despite their broad patterns of polypeptide recognition, these proteins are not interchangeable. Thus clathrin cages elicit the ATPase activity of hsc70 but not that of BiP (ref. 148), and neither DnaK nor BiP can replace prp73 in promoting targeting of RNAase A for lysosomal degradation[94]. Evidence is lacking, however, for evolution of structural features that limit recognition by individual stress-70 proteins to *bona fide* targets, as DnaK will bind eukaryotic cellular or viral proteins when they are expressed in *E. coli*[114], and BiP will recognize bacterial enzymes or virally encoded nuclear antigens when they are artificially introduced into the ER lumen by virtue of addition of an *N*-terminal hydrophobic signal sequence[151,152]. Nevertheless, target recognition by these proteins is not indiscriminate: many examples exist of authentic secretory proteins that do not seem to associate with BiP. Similarly, high-affinity BiP binding may be confined to specific domains in individual polypeptides. Thus the CH_1 domain of immunoglobulin heavy chain is necessary for stable interaction with BiP (ref. 137), and binding of BiP to influenza haemagglutinin is apparently limited to sequences in the stem domain (M. Segal, J. S. and M.-J.G., unpublished results). In these cases BiP seems to interact with sequences that form subunit interfaces because the CH_1 domain of the immunoglobulin heavy chain is the site for docking of the light chain[137], whereas the haemagglutinin trimer assembles through cooperative folding of sequences in the stem domains[3,153]. Some proteins or protein domains may lack appropriate recognition signals and never interact with BiP. Alternatively, BiP may be involved in the folding of all nascent molecules in the ER but some interactions may be too transient to be detected experimentally, possibly because BiP interacts with different segments of polypeptides with a spectrum of affinities. In support of this latter hypothesis, BiP and hsc70 display marked differences in affinity for synthetic peptides as measured in a peptide-dependent ATPase reaction[148]. In a small set of randomly chosen peptides, a range of at least 1,000-fold of Michaelis constant (K_m) values was obtained. Unfortunately, no pattern could be discerned that correlates any sequence or structural features of the peptides with their binding affinities.

In some cases (notably that of BiP and nascent secretory proteins), stress-70 proteins discriminate between folded and unfolded polypeptides, showing no propensity to associate with native protein structures. In other cases (for example DnaK with DNA replication complexes; hsc70 with clathrin cages) these proteins interact with apparently fully folded substrates and function to alter protein–protein contacts in multisubunit complexes. These schemes are not necessarily mutually exclusive, as DnaK and hsc70 also bind to unfolded polypeptides[72,114], and BiP may interact with protein components of the ER translocation system[138]. To unify these observations, Rothman[16] has suggested that the stress-70 proteins (and the other chaperones) be regarded as polypeptide-chain binding proteins (PCBs) with peptide-binding sites that can interact with chain segments only when they are part of incompletely folded structures or when they extend as loops from otherwise fully folded proteins. This hypothesis can explain how stress-70 proteins can interact with broad specificity with unfolded polypeptides whereas individual 'folded' proteins (such as clathrin light chains[92]) can use a bait of extended chain to suborn the chaperone activity of a particular stress-70 family member to a specific purpose.

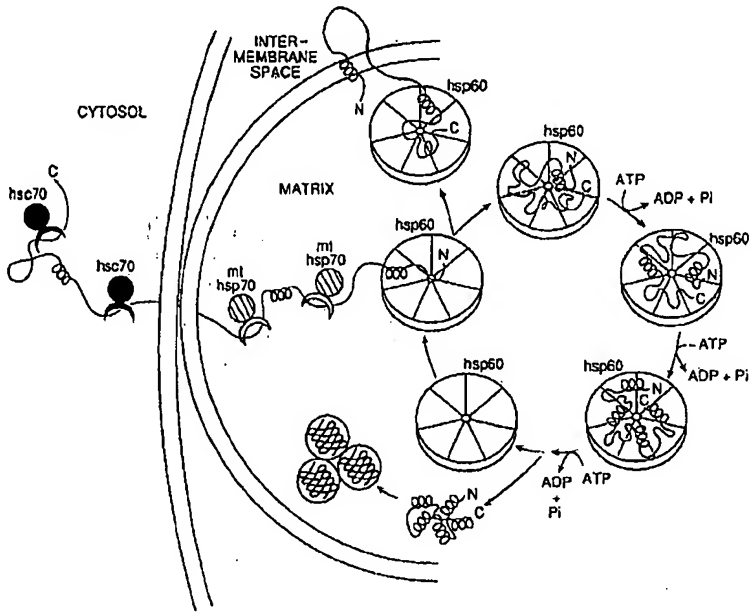

FIG. 2 Illustration of the proposed roles of hsp70 and chaperonin molecules during the import of mitochondrial precursors, their subsequent folding in the mitochondrial matrix and their reexport to the intermembrane space. The diagram is based on Fig. 1 from Neupert *et al.* (ref. 172) and in addition reflects multiple steps of folding on the chaperonin surface as suggested by Martin *et al.* (ref. 199).

Structural conservation of stress-70 proteins. Members of the stress-70 protein family have been highly conserved throughout evolution. DnaK, the single stress-70-related protein of *E. coli*, has about 50% amino-acid sequence identity with stress-70 proteins of eukaryotes[154], which are encoded by multiple *HSP70* genes that share between 50 and 95% identity at the nucleotide level[68]. Comparison of all the known amino-acid sequences of stress-70 family members reveals that the N-terminal two-thirds (about 450 amino acids) of these proteins are much more highly conserved than the C-terminal portions, suggesting a conserved domain followed by a variable region[155,156] (Fig. 3*a*). In addition, some stress-70 proteins contain short N-terminal or C-terminal extensions required for targeting to, or retention in, the appropriate cellular compartment. Thus BiP contains a cleavable N-terminal hydrophobic signal sequence specifying import into the ER[79,125] and a C-terminal tetrapeptide (for example KDEL in mammalian BiPs, HDEL in yeast BiP) that is partly responsible for retention of the protein within the ER lumen[157]. Similarly, stress-70 members located in mitochondria contain a hydrophilic N-terminal extension required for import into that organelle[141].

ATP binding and hydrolytic activity are retained by a roughly 44K, N-terminal proteolytic fragment of bovine clathrin-uncoating ATPase (hsc70), although the ATPase activity is uncoupled from its normal dependence on clathrin binding[155]. Similar N-terminal fragments are generated after proteolytic digestion of mammalian[149] and yeast[79] BiP proteins. The three-dimensional structure of the N-terminal fragment of bovine hsc70 has recently been solved to a resolution of 2.2 Å[158], revealing that the ATPase domain consists of two lobes with the nucleotide bound at the base of a deep cleft between them (Fig. 3*b*). Surprisingly, the folding topology of the hsc70 ATPase domain is nearly identical to that of the globular G actin monomer, despite there being little sequence homology between the two proteins[159]. G actin monomers contain one molecule of noncovalently bound ATP, which is hydrolysed to form bound ADP and inorganic phosphate when G actin polymerizes to

form filamentous actin. The tertiary structure of the nucleotide-binding core of the hsc70 fragment is also similar to that of hexokinase, although the remainder of the structures of the two proteins are completely dissimilar[158].

The C-terminal domain of hsc70, which has been proposed[155] to be the 'specificity' domain that couples binding of target proteins to the ATPase activity of the conserved N-terminal domain, may 'dock' onto a face of the N-terminal domain that is lined with amino-acid residues that are highly conserved between stress-70 proteins[158]. Although the structure of this portion of the molecule has not yet been determined for any stress-70 protein, two groups have now presented hypothetical models for the hsc70 C-terminal domain[160,161]. Rippman *et al.*[160] deduced a consensus secondary structure for the C-terminal domains of 33 stress-70 proteins and obtained a pattern of helices and β-strands that could be aligned with that of the α-1 and α-2 domains of the human MHC class I antigen HLA. Flajnik *et al.* found that the same domains of an MHC class I protein from *Xenopus laevis*[162] have a low amount of sequence identity with the C-terminal sequences of hsc70 and BiP proteins; they then showed that secondary structure predictions and hydropathy analyses for the corresponding regions yield very similar results if a few gaps or insertions are introduced to optimize the alignment[161]. These findings prompted both groups to model the hsc70 C-terminal domain using the known three-dimensional structure of the human class I molecule[163] (Fig. 3*c*). The putative peptide binding cleft in each of the hypothetical structures is lined with both hydrophobic and hydrophilic residues. If the peptide binding domain of stress-70 proteins does indeed closely resemble that of HLA, it is very likely that the polypeptide chain would bind in an extended conformation[164]. In fact, preliminary studies using NMR (nuclear magnetic resonance) indicate that DnaK binds a 13-residue synthetic peptide in a conformation that lacks any defined structural features[165].

A common mechanism for stress-70 action? From this abundance of disparate observations on the interaction of stress-70 proteins with their targets emerges a working model for a common mechanism for stress-70 action. Stress-70 proteins interact (probably through their C-terminal domains) with unfolded segments of polypeptide chain (Fig. 1). These unfolded segments may be presented either as nascent polypeptides emerging from the ribosome or from the lipid bilayer after membrane translocation, as sequences exposed by partial protein denaturation following an environmental stress, or as peptide loops extended from an otherwise native protein molecule. Sequence variability in the C-terminal domains of different family members may determine differences in the specificity of stress-70-peptide interactions. Although the basis for this specificity is not understood in any detail, it is likely that each stress-70 protein binds different polypeptide segments with a wide spectrum of affinities. Low-affinity binding may be reversed quickly and spontaneously, whereas release of peptide segments that are bound with high affinity may involve ATP hydrolysis mediated by the N-terminal domain of the stress-70 protein, or be effected through intervention of another cellular component (for example DnaJ, the NEM-sensitive factor, or both). Once released, the polypeptide chain has the opportunity to complete its folding by forming intramolecular interactions, or to assemble into oligomeric structures with nearby polypeptide chains, or to engage with the appropriate membrane translocation machinery or with another chaperone such as hsp60 (see below). If such interactions are not formed rapidly, stress-70 proteins, which are present at high concentration, may rebind and again stabilize the unfolded protein. During each interval of release, the polypeptide may either fold productively or form nonproductive intra- or intermolecular interactions yielding misfolded or aggregated molecules. It seems that the amino-acid sequences of wild-type proteins have evolved such that, under normal physiological conditions, productive folding or rebinding to stress-70 molecules are more likely events than misfolding. Thus wild-type polypeptides can be maintained in an assembly competent state for very long periods in the absence of their appropriate homologous or heterologous partner subunits. Similarly, cycles of binding, release and rebinding will extend the period of interaction of stress-70 proteins with polypeptides that are unable to fold productively. Sequence alterations (amino-acid substitutions, deletions or insertions), aberrant post-translational modification (for example glycosylation) or conditions of stress (high temperature, altered redox potential, decreased Ca^{2+} concentration) would perturb the normal folding pathway and increase the probability of misfolding. In such circumstances intervention by stress-70 proteins may be able to delay, but not prevent, the formation of misfolded and/or aggregated structures that are dead-ends off the folding pathway. This aberrant folding could result in occlusion of the available sites on the polypeptide for the stress-70 protein, rendering the chaperone's action less effective. Such substoichiometric binding of BiP to aggregates of nonglycosylated haemagglutinin molecules has been observed in *in vivo* experiments[135].

It is obvious that any increased probability of misfolding could be reversed by increasing the local concentration of stress-70 protein, and it is equally clear that the cell has evolved mechanisms to sense increased amounts of nascent or unfolded proteins in different cellular compartments and to respond by inducing the transcription of the appropriate stress-70 gene. For example, in *E. coli* accumulation of unfolded proteins causes increased synthesis of DnaK (and other heat-shock proteins)[113,166]. In eukaryotic cells, accumulation of unfolded proteins[76] or secretory precursors[79] in the cytosol results in induction of hsp70 and/or hsc70 proteins, whereas accumulation of unfolded proteins in the ER causes induction of BiP[78,79]. Increased concentrations of individual, constitutively expressed stress-70 proteins can be achieved by accelerating their rates of synthesis; in cases where closely related family members can perform the same or similar tasks[167], synthesis of the constitutively expressed proteins may be augmented by *de novo* induction of closely related family members. In no case do we yet understand the nature of the induction signal generated by the presence of unfolded proteins or the pathways of their transduction to the nucleus (across the ER membrane in the case of BiP).

Finally, it has been suggested that stress-70 proteins assist correct polypeptide folding not only through their 'anti-folding' function but also by disentangling malfolded or aggregated proteins using the energy released during ATP hydrolysis[87,120,168]. In cell-free translocation studies, precursors that have acquired defined structures after *in vitro* translation require unfolding for import into mitochondria[2,169-171] or ER microsomes[84,85,121]. As translocation in these systems is dependent on the presence of stress-70 proteins[84-86,121] and ATP[121,170], it was suggested that the stress-70 proteins might be supplying the unfolding activity. But it is now thought[172] that ATP hydrolysis may be required to release bound cytosolic stress-70 proteins and that the unfolding activity observed in cell-free extracts may be supplied by other components, such as the NEM-sensitive factor[86,121] or proteins present on the mitochondrial surface[172]. The only stress-70 protein for which unfolding activity has been directly demonstrated *in vitro* is DnaK which can dissolve aggregates of heat-inactivated RNA polymerase[93]. This process, which is dependent on the presence of hydrolysable ATP, requires at least stoichiometric amounts of DnaK. It is therefore possible that rather than actively unwinding the polypeptide chains in a catalytic process, DnaK might bind to peptide loops that are transiently exposed during 'breathing' of the structures of the malfolded proteins and, in a mechanism that parallels its putative role during normal folding, stabilize the polypeptide chain in a state competent for subsequent refolding to the correct conformation. ATP hydrolysis would then be required to promote release of DnaK to allow the polypeptide to continue the folding process.

The stress-90 protein family

Stress-90 proteins constitute a second major family of stress proteins (Table 1) whose members are present in all prokaryotic and eukaryotic organisms so far tested (reviewed in ref. 68). Members of the family show sequence conservation similar to that of the stress-70 family, there being greater than 40% identity between the various eukaryotic stress-90 proteins and the *E. coli* homologue HtpG. These proteins are present in high abundance under normal growth conditions, but can be further induced by heat shock or other forms of stress. They are less numerous than the stress-70 proteins, there being only one member of the family in *E. coli and D. melanogaster*, and two members in *S. cerevisiae* that differ in their constitutive level and degree of inducibility[68]. Vertebrate cells contain an additional stress-90 protein, variously name grp94 (ref. 173), ERp99 (ref. 174) or endoplasmin[175], which like BiP in the stress-70 family is synthesized as a precursor that contains an N-terminal signal sequence for ER translocation and a C-terminal KDEL tetrapeptide.

The cytosolic stress-90 proteins, whose apparent M_rs vary from 87-92K, associate with a diverse range of cellular proteins including retroviral transforming proteins, steroid hormone receptors, cellular protein kinases, actin and tubulin (reviewed in ref. 68). The common feature of these interactions seems to be the stabilization of the target proteins in an inactive or unassembled state. Thus the association of monomeric hsp90 (and an unidentified 50K phosphoprotein) with $pp60^{src}$ immediately after its synthesis stabilizes the transforming protein in an inactive state until it reaches its appropriate destination at the plasma membrane[176,177]. Concommitant with its release from association with hsp90, $pp60^{src}$ is phosphorylated on tyrosine, inserted into the plasma membrane, and activated as a kinase. Hsp90 is involved both in the initial folding of steroid hormone receptors[178] and in subsequent modulation of their DNA binding and transcriptional regulatory activities[179]. Thus, aporeceptors synthesized in the absence of hsp90 lack transcriptional enhancement activity and responsiveness to hormonal activation. Under normal conditions, newly synthesized aporeceptors form a complex with a dimer of hsp90 in which the receptor is stabilized in a partially unfolded conformation that is unable to bind DNA (ref. 180). Binding of steroid hormone promotes dissociation of hsp90 from the complex and allows the receptor to bind DNA. When hsp90 is removed from the complex using high salt or temperature, the interaction of the receptor with DNA is unregulated, occurring in the presence or absence of hormone. Although binding of hsp90 has been mapped to a stretch of about 80 residues in the hormone binding site on the steroid receptor[180-182], the features that determine the specificity of binding and release of target polypeptides by members of the stress-90 family are not understood. The hsp90 binds ATP in a cation-dependent manner and undergoes autophosphorylation[183]. But by contrast to stress-70 proteins and the chaperonins (see below), stress-90 proteins do not seem to catalyse the hydrolysis of ATP.

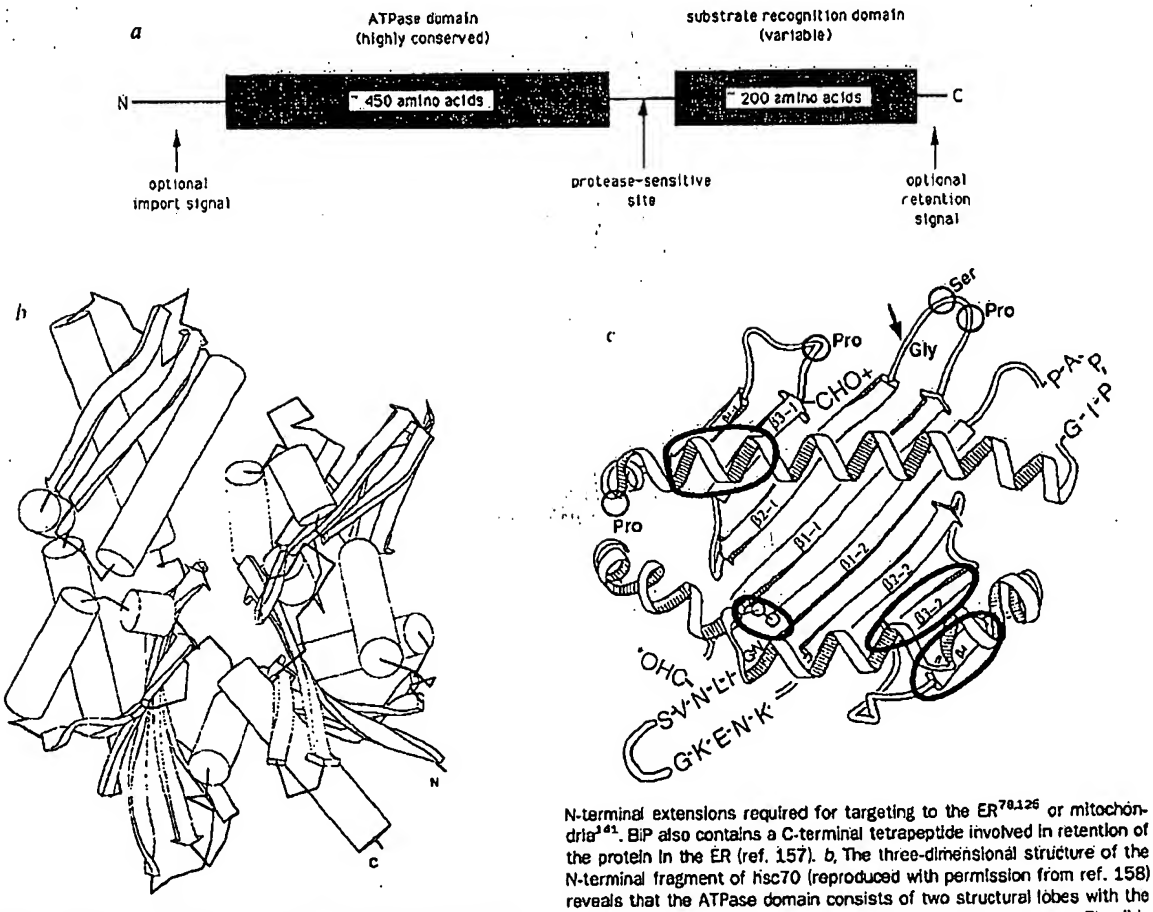

FIG. 3 Structure of stress-70 proteins. *a*, A linear diagram of a generalized stress-70 protein indicating two major domains: a highly conserved N-terminal domain that has ATPase activity and a less conserved C-terminal peptide-binding domain[155]. Some stress-70 proteins also contain short N-terminal extensions required for targeting to the ER[78,126] or mitochondria[141]. BiP also contains a C-terminal tetrapeptide involved in retention of the protein in the ER (ref. 157). *b*, The three-dimensional structure of the N-terminal fragment of hsc70 (reproduced with permission from ref. 158) reveals that the ATPase domain consists of two structural lobes with the nucleotide bound at the base of a deep cleft between them. *c*, Possible structure of the C-terminal peptide binding domain of hsc70 (reproduced with permission from ref. 161) based on the known three-dimensional structure of the human MHC class I antigen HLA (ref. 163).

Nothing is known about the function of grp94, one of the most abundant proteins resident in the ER lumen. Like BiP, grp94 is induced by the accumulation of unfolded proteins in the ER (ref. 78), suggesting that it may function with BiP to assist the assembly of nascent polypeptides. Several abundant ER-resident proteins, including grp94 and BiP, are high capacity, low-affinity Ca^{2+}-binding proteins[184], but the functional significance of this property is not understood.

The GroEL/chaperonin family

The term chaperonin was suggested by Ellis[81] to describe a class of molecular chaperones that are homologous in structure to *E. coli* GroEL. Members of this protein family are present in all prokaryotes and in those organelles of eukaryotic cells, such as mitochondria and chloroplasts, that have a probable endosymbiotic origin (Table 1). These proteins, which have been renamed chaperonin-60 (ref. 82), are large oligomers composed of 14 subunits each about 60K, arrayed as two stacked rings of seven subunits[185,186]. In *E. coli*, GroEL interacts functionally in an ATP-dependent manner with GroES (chaperonin-10), a roughly 10K polypeptide that forms a single ring of seven subunits and is the second product of the GroE operon (reviewed in ref. 96). In the absence of unfolded protein substrates, the inherent ATPase activity of GroEL is inhibited by GroES[187,188]. Mitochondria of mammalian cells contain a polypeptide that is structurally and functionally homologous to GroES (ref. 189, and P. Vūtanen, unpublished results).

Remarkably, the general features of the interactions of chaperonin-60 molecules with their target polypeptides are very similar to those of stress-70 proteins, despite great differences in their sequences and oligomeric structures (for recent reviews and references see 67, 72, 96, 190–192). Thus both types of chaperones are highly abundant proteins whose rate of synthesis can be further induced by environmental stresses such as heat shock. Members of both families have been implicated in the assembly of nascent protein subunits into macromolecular structures, as well as in a number of other fundamental cellular processes. Chaperonin-60 molecules, like stress-70 proteins, bind ATP with high affinity and have weak ATPase activity and both types of proteins in some circumstances function together with other heat-shock proteins or cellular factors. Most importantly, both seem to act on their targets by stabilizing the conformation of folding intermediates, thereby preventing the formation of aberrrant structures and directing the polypeptides down biologically productive assembly pathways.

GroEL. In *E. coli*, GroEL and GroES are abundant heat-shock proteins that are also required for viability under normal growth conditions[73]. *GroE* mutants have phenotypes reminiscent of those of DnaK mutants[72]. Thus mutants lacking either GroEL or GroES have reduced rates of DNA and RNA synthesis, are blocked in cell division at nonpermissive temperatures, and show a reduction in overall protease activity. The GroE proteins are also required for bacteriophage morphogenesis in *E. coli*. Overproduction of both GroEL and GroES, but not of either alone, can suppress temperature-sensitive mutations in a large number of different genes[193] apparently by promoting the correct folding or assembly of the mutant polypeptides. Like stress-70 proteins, the GroE proteins also have a role in secretion. In bacterial cell-free protein translocation reactions, GroEL binds newly synthesized secretory precursors stabilizing them for membrane transit[24]. *In vitro*, GroEL forms complexes with unfolded precursors of several secretory proteins including β-lactamase, proOmpA and prePhoE[24,26]. *In vivo*, GroEL is required for export of β-lactamase but not other secretory precursors[194], perhaps because other prokaryotic chaperones such as trigger factor and secB function in its place[26]. Finally, overproduction of GroEL in *E. coli* can facilitate the export of lacZ hybrid proteins[115].

Insight into the mechanism of action of the *E. coli* GroE proteins has come from studies of their role in promoting the folding and/or assembly of a number of enzymes, including prokaryotic ribulose bisphosphate carboxylase (Rubisco)[82,188,195,196], pre-β-lactamase[197], citrate synthase[198], dihydrofolate reductase[196,199] and rhodanese[199,200], and of GroEL itself[201]. Partially folded protomers of these proteins form stable binary complexes with GroEL, in a process that competes both with biologically unproductive aggregation of the polypeptide chains and with their spontaneous refolding. GroEL does not interact with native proteins or with irreversibly denatured and aggregated molecules, but rather binds to labile folding intermediates likely to correspond to 'molten globules'[14,15] or 'compact intermediates'[13]. In the absence of GroES, hydrolysis of ATP by GroEL promotes the discharge of the binary complex to release partially folded but catalytically inactive polypeptides. Whether release results in the generation of native, enzymatically active molecules depends on the nature of the polypeptide substrate and is related to the propensity of each polypeptide chain to fold spontaneously under the reaction conditions employed. If GroEL is available for rebinding after release, aggregation or continued folding of the polypeptides will be inhibited or delayed[199]. In the presence of GroES, ATP hydrolysis-dependent folding occurs at the surface of GroEL through intermediate conformations that are progressively more compact but still enzymatically inactive[199]. Finally, the polypeptide is released from the complex in a form that is apparently committed to completion of folding to the native state. In every case studied the overall effect of the coordinated action of the two GroE proteins is to increase the efficiency of refolding compared with that of the spontaneous process. But how the presence of the chaperonins influences the rate of the folding reaction varies significantly from protein to protein. Thus the rate of folding of Rubisco is enhanced 10-fold relative to the spontaneous process[188], whereas the rates of folding of citrate synthase[198] and pre-β-lactamase[197] are unchanged, and those of DHFR and rhodanese[199] are decreased. These differences are likely to be related to how the specific interaction between the polypeptide chain and the chaperonin promote or interfere with rate-limiting intramolecular interactions that normally take place during the spontaneous folding process.

No more than one or two molecules of unfolded polypeptide are bound to each oligomeric assembly of 14 GroEL molecules[26,197,199]. This stoichiometry might suggest that the GroEL protomers in each heptameric ring, or in the tetradecamer, interact to form a single binding site. Alternatively, steric hindrance could limit the access of more than one or two protein molecules to 14 identical sites located in or near the hole in the centre of the doughnut-shaped structure[185,186] (Fig. 4). A polypeptide could then be bound at up to 14 sites, each of which is capable of interacting with one of the multiple recognition motifs that may be exposed on a partially folded polypeptide. This second possiblity is compatible with the suggestion that, in the presence of GroES, the folding of DHFR and rhodanese on the surface of GroEL occurs by progressive, ATP hydrolysis-dependent release of different portions of the bound polypeptide[199]. The role of GroES would then be to modulate or coordinate the ATPase activity of each GroEL protomer, perhaps to prevent all sites discharging simultaneously leading to premature release of an only partially folded molecule[199]. Overall the result of the interaction between GroEL and GroES would be to prolong contact between the chaperonin complex and its substrate as long as the polypeptide exposes structures recognized by GroEL.

Plastid Rubisco subunit binding protein (RBP). The chaperonin-60 present in chloroplasts of higher plants is a nuclear-encoded protein first identified because of its involvement in the assembly of the hetero-oligomeric plant Rubisco[81,191,192]. In contrast to GroEL and mitochondrial hsp60s, each of which contain only one type of 60K subunits, RBP consists of two distinct but related subunits, α (61K) and β (60K), probably arranged as two layers of seven monomers each[81]. The wheat Rubisco small

subunit expressed in *E. coli* associates with GroEL, providing evidence for functional homology between GroEL and wheat RBP, which have 46% identity in amino-acid sequence. As several polypeptides associate with RBP after import into isolated chloroplasts[25] it seems likely that further studies will demonstrate the involvement of RBP in the assembly of plastid macromolecules other than Rubisco.

Mitochondrial hsp60. Mitochondria from *Tetrahymena*, yeast, maize, *Xenopus* and human cells also contain proteins that are related structurally and immunologically to the GroEL protein[185,202]. A nuclear gene (*HSP60* or *MIF4*) encodes the mitochondrial homologue in *S. cerevisiae* (hsp60)[203], which displays 54% and 43% amino-acid identity, respectively, with GroEL and the α component of chloroplast RBP. Hsp60 is expressed constitutively and is localized in the mitochondrial matrix[204]. In cells expressing a mutant (*mif4*) form of hsp60, subunits of mitochondrial enzymes fail to assemble into the appropriate macromolecular complexes despite being translocated into the mitochondrial matrix and undergoing normal processing in that compartment[204]. This is the case not only for proteins normally destined for the matrix, such as the β-subunit of F_1-ATPase and hsp60 itself[205], but also for proteins with complex presequences, such as cytochrome b_2 and the Rieske Fe/S protein, whose final destination is the intermembrane space. These latter proteins accumulate as incompletely processed import intermediates[204]. Hsp60 has been directly implicated in folding of proteins in the mitochondrial matrix[206]. When assembly of precursors imported into wild-type mitochondria is arrested by depletion of ATP, by NEM treatment, or at low temperature, the unfolded polypeptides are associated with hsp60 in a high-M_r complex. Addition of ATP allows at least partial refolding of the polypeptides but does not promote their release from the complex. An unidentified factor seems to be required for the release reaction. Fig. 2 illustrates our current view of the role played by stress-70 and chaperonin molecules during the translocation and folding of polypeptides imported into mitochondria.

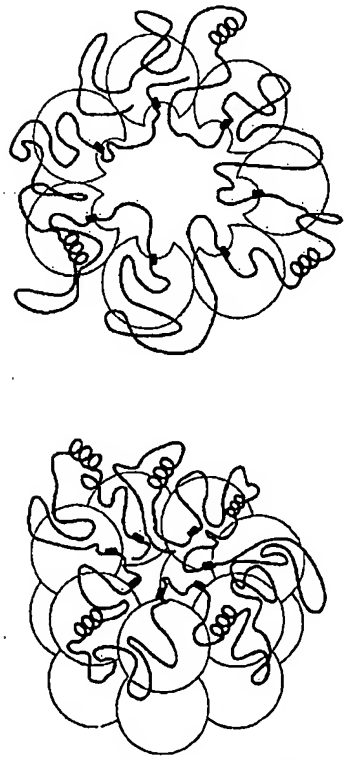

FIG. 4 Hypothetical model of the GroEL chaperonin structure reflecting multiple binding sites for a single polypeptide chain. The oligomeric structure is shown in views from the top and the side. The small corkscrews represent α-helices that may be recognition elements for binding to GroEL; the larger corkscrews represent general secondary structures in the bound polypeptide.

Mechanism of action of chaperonins? Although the broad features of the interaction of chaperonins with their polypeptide substrates have been illuminated by *in vitro* studies with GroEL and GroES (see above), many questions remain concerning the molecular details of the mechanism by which chaperonins promote protein folding and assembly. The quaternary structure of chaperonin oligomers has been revealed by electron microscopy[185,186], but nothing is known about the tertiary structures of chaperonin-60 or chaperonin-10 protomers or how they interact in the complex. Nor do we understand how chaperonins recognize their target polypeptides. The available evidence suggests that chaperonin-60 molecules bind to structural elements that are displayed by compact folding intermediates of a broad range of polypeptide substrates but absent or inaccessible in the native or aggregated forms of these proteins. The nature of these structural elements is not yet known, although preliminary experiments using NMR indicate that GroEL binds synthetic peptides that have the potential to form amphipathic α helices[207]. It will be important to learn whether chaperonin-60 molecules, like stress-70 proteins, display marked sequence specificity for binding. If so, recognition motifs on a folding protein may have evolved with a hierarchy of affinities that directs their order of release from the chaperonin complex. If so, rather than facilitating folding in a merely permissive fashion, the interaction between chaperonin and substrate may influence the pathway and the kinetics of the folding process. Finally, we need to understand the role that ATP hydrolysis plays in the folding and/or release of bound polypeptides and the manner in which chaperonin-10 regulates both this ATPase activity and the release reaction.

Roles of chaperonins and stress-70 proteins

Although stress-70 proteins and chaperonins share many common features in their modes of action, they do not perform interchangeable roles. Despite their coexistence in bacteria and in mitochondria and plastids of eukaryotic cells, each type of chaperones is independently essential for cell viability.

In mitochondria, both types of chaperones interact with unfolded or prefolded molecules: hsp70 molecules associate with imported polypeptides even before their translocation is completed[147], whereas hsp60 molecules become involved at a later stage[90,204]. It is therefore possible that stress-70 molecules interact with less folded structural elements (perhaps segments of extended polypeptide chain[16]), whereas chaperonin-60 molecules associate with structural features common only to folding intermediates[196]. Interestingly, two-dimensional NMR studies reveal that a synthetic peptide is bound by DnaK in an extended form, and by GroEL in an α-helical conformation[165].

Third, the two types of chaperones seem to play different roles during the process of polypeptide folding. Thus stress-70 molecules are thought to stabilize unfolded forms of their substrates; folding is envisaged as occurring after release from the chaperone. On the other hand, at least partial folding of polypeptides may take place on the surface of chaperonin-60 oligomers[82,199,206]. This distinction may be a consequence of the dramatic difference in the oligomeric state of the two types of molecules. Stress-70 proteins are thought to contain a single peptide-binding site and to associate as monomers with their target polypeptides[91]. Therefore, any folding step that is inhibited by chaperone binding must occur after dissociation of the complex. By contrast, the functional form of the chaperonin-60 molecule is an oligomer of 14 subunits, which apparently binds no more than 1 or 2 target polypeptides[26,197,199]. Multivalent binding of a protein to the chaperonin-60 oligomer would allow release and partial folding of one portion of the chain while the polypeptide remains attached at other sites. The extent of folding of any protein on the chaperonin-60 surface might then depend on the role in the folding process of the portion of the chain that is bound with highest affinity.

Fourth, although stress-70 proteins such as DnaK and hsc70 are known to interact both with unfolded polypeptides and with protein oligomers (such as replication complexes or clathrin triskelions), evidence is lacking for any *in vivo* role for chaperonins in the rearrangement of oligomeric complexes.

Finally, although stress-70 and chaperonin-60 molecules function alongside each other in bacteria and in the matrix of mitochondria and plastids, chaperonins have not been identified in other compartments of eukaryotic cells. Whether stress-70 family members manage alone in supporting polypeptide folding and assembly in those compartments, or whether other proteins fulfil the function of the chaperonins remains to be determined.

Future directions

We now appreciate that chaperones are involved at all stages of cellular metabolism, during protein biosynthesis and maturation, in protection from environmental stress, in rearrangements of cellular macromolecules during functional cycles of assembly and dissassembly, and finally in targeting proteins for degradation. Much progress has been made in characterizing chaperones that are members of three families of major stress proteins, and in identifying a number of unrelated proteins that also regulate or facilitate polypeptide folding in the cell. The major challenge now lies in elucidating the specific molecular mechanisms by which chaperones recognize their target proteins and promote, inhibit or reverse folding and assembly.

Although acceptance of the involvement of chaperones has required some revision of long-held views about the spontaneity of the process of protein folding, there was less resistance to a role during folding for enzymes such as PDI and the PPIases. Despite advances in our understanding of their mechanisms of action, important questions remain to be answered about the *in vivo* function and substrates of these enzymes and of a variety of newly discovered related proteins. □

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ARTICLES

Ridges, hotspots and their interaction as observed in seismic velocity maps

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A new global S-wave velocity model reveals that although mid-ocean ridges and hotspots are both underlain by low-velocity anomalies in the mantle, these have distinctly different structures. This implies that there are differences between the upwelling mechanisms under ridges and under hotspots. The velocity model also shows that there may be interactions between ridges and hotspots near Afar and St Helena.

RIDGES and hotspots are two main forms of upwelling from the interior of the Earth and are essential features of global tectonics. In ocean basins, they are the dominant modes of igneous activity, and are known to produce magmas of differing, although sometimes overlapping, geochemistry[1]. The mechanisms operating below these features have been inferred from surface observations, including geochemical, topographic and geoid data, and theoretical models based on gross structure and evolution of the mantle[2–5]. Seismic data provide critical information for understanding these features from maps of three-dimensional (3D) structure. On a local scale, seismic techniques have been successful, for example, in constraining the size of magma chambers under ridges[6], but such studies are restricted to particular areas where dense observations have been made.

We have obtained a new global, 3D S-wave velocity model for the upper mantle by analysis of travel times of long-period Love and Rayleigh waves (75–250 s) using ~18,000 seismograms. It is now becoming possible for global seismic studies to detect seismic velocity anomalies caused by various tectonic features (as opposed to purely long-wavelength variations), and to make comparisons between regions in order to understand the underlying mechanisms. Details of the data analysis and S-wave velocity structure are reported elsewhere[7,8]. Here we focus on S-wave velocity variation under ridges and hotspots.

Long-wavelength features of the new model are similar to previous global, 3D seismic models[9–14]. But previous models were represented by spherical harmonic expansion only up to angular degrees 6–10, with horizontal resolution lengths of 4,000–6,000 km. The chief advance of our model, which is expanded up to degree 36, is a considerable improvement in the

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Exhibit B

Mini-review

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Influence of molecular and chemical chaperones on protein folding

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Abstract Protein folding inside the cell involves the participation of accessory components known as molecular chaperones. In addition to their active participation in the folding process, molecular chaperones serve as a type of 'quality control system', recognizing, retaining and targeting misfolded proteins for their eventual degradation. It is now known that a number of human diseases arise as a consequence of specific point mutations or deletions within genes encoding essential proteins. In many cases these mutations/deletions are not so severe as to totally destroy the biological activity of the particular gene product. Rather, the mutations often result in only subtle folding abnormalities which lead to the newly synthesized protein being retained at the endoplasmic reticulum by the actions of the cellular quality control system. In this short review article we discuss our recent studies showing that the protein folding defect associated with the most common mutation in patients with cystic fibrosis can be overcome by a novel strategy. As shown in the paper by Brown et al in this issue (Brown et al 1996), a number of different low molecular weight compounds, all known to stabilize proteins in their native conformation, are effective in rescuing the processing defect of the mutant cystic fibrosis transmembrane conductance regulator protein. We then discuss how these same compounds, which we now call chemical chaperones, also may prove to be effective in correcting a number of other protein folding abnormalities which constitute the underlying basis of a large number of different human diseases.

MOLECULAR CHAPERONES AND QUALITY CONTROL

The fidelity of protein folding and assembly inside the cell is mediated by a class of proteins now commonly termed molecular chaperones (reviewed by Ellis and van der Vies 1991; Georgopoulos and Welch 1993). Situated throughout various cellular compartments, molecular chaperones interact with nascent polypeptides during the course of their synthesis and/or translocation into cellular organelles. All of the available evidence indicates that many of the chaperones can distinguish between the native and non-native states of a polypeptide (Palleros et al 1991; Sadis and Hightower 1992). For most polypeptides the interaction with molecular chaperones is transient; once properly folded the newly synthesized protein no longer represents a substrate for chaperone interaction. In contrast, proteins unable to achieve a thermodynamically stable, properly folded conformation exhibit relatively long-lived interactions with one or more members of the chaperone family. This capacity for discriminating between properly folded versus misfolded proteins has led to the idea that the chaperones provide the cell with a type of 'quality control system', recognizing, retaining and targeting misfolded proteins for degradation (reviewed by Hammond and Helenius 1995). The role of different chaperones in maintaining quality control is best illustrated by examining the early stages of protein maturation within the secretory pathway. As an unfolded polypeptide enters into the lumen of the endoplasmic reticulum (ER) it interacts with, and is stabilized by, its interaction with Bip and/or calnexin, and perhaps other members of the molecular chaperone family. Following completion of its folding

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Table 1 Diseases involving defective protein folding

Disease	Protein	Problem
α_1-Antitrypsin deficiency	α_1-Antitrypsin	Trafficking/aggregation
Cystic fibrosis	CFTR	Trafficking
Familial hypercholesterolemia	LDL Receptor	Trafficking
Glanzmann's thrombasthemia	Integrin	Trafficking
Leprechaunism	Insulin receptor	Trafficking
Leukocyte adhesion deficiency	Integrin	Trafficking
Retinitis pigmentosa	Rhodopsin	Trafficking
Tay-Sachs	β-Hexosaminidase	Trafficking
Amyotrophic lateral sclerosis	Superoxide dismutase	Misfolding
Cancer	p53 (other oncogenes/ tumor suppressors?)	Misfolding
Marfan syndrome	Fibrillin	Misfolding
Osteogenesis imperfecta	Procollagen	Misfolding
Alzheimer's	β-amyloid	Aggregation
Cataracts	Crystallins	Aggregation
Familial amyloidosis	Transthyretin	Aggregation
Scrapie (Mad Cow) Creutzfeldt-Jakob Familial insomnia	Prion	Aggregation

or assembly the newly synthesized protein exits the ER and moves further along the secretory pathway. Proteins that are unable to adopt a stable conformation, due for example to specific mutations or aberrant glycosylation, are prevented from further movement out of the ER by the actions of one or more of the molecular chaperones. Over time these abnormally folded proteins are targeted to a degradative pathway. Thus, by acting as a type of molecular filter, the ER quality control machinery allows properly folded proteins to pass through the first stage of the secretory pathway, but retains those proteins which are misfolded and therefore unlikely to possess biological activity.

THE QUALITY CONTROL MACHINERY AND DISEASE

Abnormalities in protein folding constitute the molecular basis for many human diseases (Table 1; see review by Thomas et al 1995). Genetically inherited diseases are often characterized by specific point mutations or deletions which give rise to protein products which fail to achieve their properly folded state. In some cases the mutations are so severe as to render the gene product biologically inactive. In other cases, however, the mutations are relatively minor and result in the protein exhibiting only a partial loss of its normal activity. Thus, even though still exhibiting some biological activity, these mutant proteins are not delivered to their correct site either inside the cell or secreted out of the cell. One example of a disease involving abnormal protein trafficking is α_1-antitrypsin deficiency. Here a deficiency in the secretion of this protease inhibitor is the basis of metabolic liver disease in children, and is associated with both cirrhosis and emphysema in adults (reviewed in Teckman and Perlmutter 1995). A single point mutation within the gene encoding α_1-antitrypsin inhibitor significantly reduces the amount of the protein which is secreted out of the cell. Patients with the so-called Z mutation in α_1-antitrypsin secrete only about 15% of the newly synthesized molecules, with the remainder of the Glu 342→ Lys protein never leaving the ER. The secreted protein is apparently functional as a protease inhibitor (Yu et al 1995). The retention of the majority of the mutant protein in the ER is mediated, at least in part, by the calnexin chaperone (Le et al 1994). While some of this retained material is eventually degraded, the remainder tends to form aggregates in the ER. Over time the accumulation

of these aggregates interferes with the normal activities of the hepatocyte. In addition, the corresponding decline of the protein in the plasma is thought to contribute to the development of pulmonary emphysema.

A second example where abnormal protein folding appears to constitute the basis of the disease state is cystic fibrosis (CF). A number of mutations within the coding region for the cystic fibrosis transmembrane conductance regulator (CFTR) protein have been described and shown to interfere with the maturation of the protein (Cheng et al 1990; reviewed in Riordan 1993). The wild-type CFTR protein is found at the plasma membrane and is believed to function as a cAMP-activated chloride channel. The predicted topology of the protein includes 12 membrane-spanning domains, with the vast majority of the protein present within the cytoplasm (Riordan et al 1989). The newly synthesized CFTR protein is found in the ER in a complex with at least two molecular chaperones: calnexin within the ER lumen and Hsp70 within the cytoplasm (Yang et al 1993; Pind et al 1994). Subsequent movement of the protein out of the ER to the plasma membrane is accompanied by the release of its two chaperone escorts. The most common mutation observed in patients with CF (over 70%) is a deletion of a phenylalanine residue at position 508 of the CFTR protein (ΔF508 CFTR). This single-point mutation results in a failure of the protein to traffic to the plasma membrane and hence, an inability of the cells to transport chloride in response to increases in intracellular cAMP levels (Cheng et al 1990). Instead, almost all of the ΔF508 CFTR mutant is retained at the level of the ER, in a complex with both calnexin and Hsp70, until its eventual degradation.

The processing defect associated with the ΔF508 CFTR mutant is temperature sensitive. Reducing the temperature of cells expressing ΔF508 CFTR below 30°C results in a portion of the mutant protein trafficking to the plasma membrane. These cells now appear competent for forskolin-dependent chloride transport, even when shifted back to 37°C (Denning et al 1992). This restoration of chloride transport activity is only transient. When the cells are left at 37°C, the mutant protein present at the plasma membrane is eventually degraded, with the kinetics of its turnover similar to that observed for the wild-type CFTR protein.

Thus, the most prevalent mutations observed for the CFTR protein as well as the α_1-antitrypsin inhibitor result in only subtle folding abnormalities that, for the most part, do not grossly interfere with their proper function. Rather, the problem appears to be a failure of the newly synthesized mutant proteins to exit from the ER and move to their proper locale, likely due to the actions of one or more components of the quality control machinery.

CORRECTING PROTEIN FOLDING DEFECTS ASSOCIATED WITH DISEASE

The observation that the ΔF508 CFTR protein can function as a chloride channel if it successfully transits to the plasma membrane, raises the possibility that strategies other than gene therapy might prove to be effective for the treatment of the disease. Owing to its temperature sensitivity in folding, we examined whether compounds known to stabilize proteins against thermal treatments might correct the folding defect of the ΔF508 CFTR mutant. Examples of such compounds include glycerol, deuterated water, the organic solvent dimethylsulfoxide and, finally, the cellular osmolyte trimethylamine N-oxide. As shown in the paper by Brown et al in this issue (Brown et al 1996) and summarized in Figure 1, these different protein stabilizing agents were effective in correcting the processing defect associated with the maturation of the ΔF508 CFTR protein. Moreover, following treatment with these various compounds, the cells expressing the mutant CFTR protein now exhibited forskolin-dependent chloride transport, almost equal in magnitude to that observed for cells expressing the wild-type form of the CFTR protein. Similar results using glycerol to correct the ΔF508 CFTR folding defect recently have been reported by Sato et al (1996).

These observations raise the possibility that a similar strategy might prove to be effective for correcting other protein folding abnormalities associated with specific diseases. Like the situation with the Z variant of α_1-antitrypsin or the ΔF508 CFTR mutation, many naturally occurring mutations interfere with the normal trafficking of medically important proteins. Examples include the low density lipoprotein receptor, the insulin receptor, B-hexosaminidase and rhodopsin (reviewed by Thomas et al 1995). In each case specific mutations result in a failure of the newly synthesized proteins to exit the ER, likely due to their retention by the actions of the quality control machinery. Whether these different mutant proteins, if delivered to their correct location in the cell, will function like their wild-type counterparts has not been established. Nevertheless, it will be interesting to see whether the different protein stabilizing agents that rescued the maturation of the mutant CFTR protein also can correct the folding and/or trafficking defects of these other medically important mutated proteins. If so, will we then observe a correction of the abnormal cellular phenotype?

Yet other diseases involving protein misfolding include neurological disorders such as Alzheimer's and prion related disease (reviewed by Prusiner and DeArmond 1994; Selkoe 1994; Kenward et al 1996). Here the problem does not appear to be one of trafficking or retention of the newly synthesized proteins by components of the

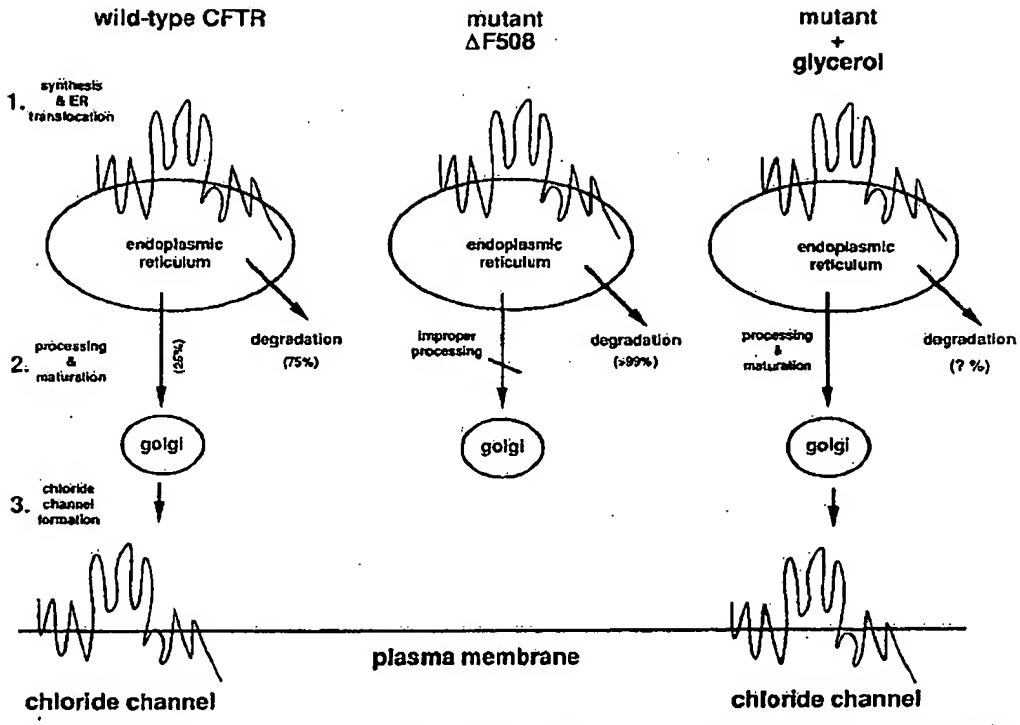

Fig. 1 Chemical chaperones like glycerol correct the processing defect associated with the ΔF508 CFTR protein. **Wild-type CFTR**: The wild-type cystic fibrosis transmembrane conductance regulator (CFTR) protein is synthesized and inserted into the membrane of the endoplasmic reticulum (ER). Although still not fully understood, only about 25% of the newly synthesized wild-type protein moves to the Golgi complex on its way to the plasma membrane where it functions as a cAMP-dependent chloride channel. The remainder of the wild-type protein (approx. 75%) is degraded. **Mutant ΔF508**: The newly synthesized ΔF508 CFTR mutant also is observed to be inserted into the membrane of the ER. In contrast to the wild-type protein, however, little or none of the protein is ever observed to move to the Golgi complex, likely being retained at the ER via the actions of the Hsp73 and calnexin chaperones. Over time the mutant protein is degraded. These cells fail to exhibit cAMP-mediated chloride transport. **Mutant + glycerol**: Incubation of cell expressing the ΔF508 CFTR protein in the presence of 0.5–1.25M glycerol results in a portion of the mutant protein undergoing proper processing, including movement through Golgi complex and localization to the plasma membrane. Like the situation with the wild-type protein, a significant portion of the newly synthesized protein, even in the presence of glycerol, likely is targeted for degradation. Cells expressing the mutant protein in the presence of glycerol now exhibit cAMP-stimulated chloride transport.

quality control machinery. Rather, mature proteins which have reached their correct site in the cell (e.g. plasma membrane) tend to misfold following their internalization, likely at some point during the degradative pathway. As a result, the partially degraded and misfolded proteins have a tendency to precipitate. These precipitates eventually form insoluble aggregates which then are resistant to further proteolytic digestion. The insoluble protein aggregates, which over time accumulate to rather high levels, are thought to interfere with proper neuronal function, leading to cognitive impairment. For example, the formation of the pathogenic prion protein (PrP^{Sc}) is thought to involve a conformational change in which one or more α-helices of the wild-type PrP^{C} protein unfolds and then refolds into a β-sheet, resulting in its aggregation inside the cell (Pan et al 1993; Cohen et al 1994). In the case of infectious prion disease, exogenously introduced PrP^{Sc} (e.g. ingestion of a food source containing the prion protein) somehow finds its way into the brain and apparently interacts with, and drives the conversion of, endogenously synthesized PrP^{C} into the PrP^{Sc} isoform. In inherited or familial cases, a mutation in the PrP gene has been suggested to destabilize the PrP^{C} protein, resulting in a portion of the protein to adopt the mutant PrP^{Sc} conformation spontaneously (Cohen et al 1994). Once formed, PrP^{Sc} then acts as a template to further propagate the conversion of PrP^{C} into the pathogenic PrP^{Sc} isoform.

Here again we have found that various protein stabilizing agents can apparently correct the protein folding abnormality associated with the formation of the pathogenic prion protein. For example, a neuronally derived cell line has been established which propagates the production of the scrapie prion protein. These so-called ScN2a cells convert approximately 10% of the newly synthesized PrP^{C} into the PrP^{Sc} isoform. Tatzelt et al (1996)

Table 2 Common cellular osmolytes

I. Amino acids & derivatives	II. Carbohydrates	III. Methylamines
Alanine	Arabitol	Betaine
Glutamic acid	Glycerol	Glycerophosphorylcholine
Proline	Mannitol	Sarcosine
γ-Aminobutyric acid	Mannose	Trimethylamine N-oxide
Taurine	Sorbitol	
Sucrose		
Trehalose		
Myo-inositol		

have recently shown that the addition of different protein stabilizing agents, including glycerol DMSO and trimethylamine N-oxide, to the ScN2a cells effectively blocked the conversion of PrP^{c} into PrP^{Sc}. The protein stabilizing agents did not appear to affect the existing population of the insoluble PrP^{Sc} protein. Rather they interfered only with the conversion of newly synthesized PrP^{c} into PrP^{Sc}, probably at some point during the early stages of internalization and degradation of PrP^{c}. We suspect that glycerol and these other low molecular weight compounds act by stabilizing PrP^{c} in its α-helical conformation. This would then reduce the propensity of the partially degraded protein to re-fold into the β-sheet conformation upon encountering the existing population of PrP^{Sc}. It will be interesting to see whether these same compounds can also interfere with the formation of amyloid deposits associated with Alzheimer's disease, a process also thought to result from the misfolding of the amyloid β-precursor protein.

CELLULAR OSMOLYTES ACT AS CHEMICAL CHAPERONES TO COUNTERACT PROTEOTOXIC ENVIRONMENTS

Owing to their positive influence on protein folding we have begun to refer to the above described protein stabilizing agents (e.g. glycerol) as 'chemical chaperones'. Like the protein molecular chaperones, the different chemical chaperones obviously do not provide any direct information for the folding process. Rather, they seem to influence the rate or fidelity of the folding reaction, probably by stabilizing the properly folded form of the polypeptide (reviewed in Schein 1990). For example, polyhydric alcohols (or polyols) like glycerol tend to be excluded from the immediate vicinity of a polypeptide. As a result, at relatively high concentrations glycerol will act to increase the relative hydration around the polypeptide. In response, the polypeptide will tend to decrease its relative surface area by an increase in its self-association or tighter packing. This type of hydrophobic effect in the presence of glycerol acts to enhance the stability of the protein and thereby reduce its potential to unfold in response to thermal or chemical treatments (Gekko and Timasheff 1981a, 1981b).

Nature has developed her own set of chemical chaperones to help organisms deal with adverse changes in their environment which might lead to protein denaturation. For example, when exposed to hyperosmotic stress, cells in the kidney maintain their osmotic balance by accumulating a variety of different low molecular weight organic solutes, referred to as cellular osmolytes (Table 2; reviewed in Yancey et al 1982; Somero 1986; Garcia-Perez and Burg 1991). Cellular osmolytes are comprised of three classes of organic compounds:

1. Carbohydrates including glycerol, sorbitol, arabitol, myo-inositol and trehalose
2. Free amino acids and/or amino acid derivatives such as glycine, alanine, proline, taurine and γ-amino butyric acid
3. Methylamines such as betaine, trimethylamine N-oxide (TMAO), and glycero-phosphorylcholine.

Members of the first two groups often are referred to as 'compatible osmolytes' owing to the fact that they can accumulate within the cell to rather high concentrations without significantly perturbing protein function. In contrast to inorganic ions which can have adverse effects on the structure and function of proteins, compatible osmolytes not only help deal with the problem of changes in water availability, but also act to stabilize proteins and perhaps other macromolecules in their native conformation. 'Counteracting osmolytes', represented by the methylamines, are produced to offset the protein denaturing effects of urea. For example, in the tissues of some saltwater organisms (e.g. sharks), as well as in the mammalian renal medulla, urea concentrations can reach dangerously high levels. To counteract this potential protein denaturing environment, methylamine concentrations are usually increased to about half the concentration of that observed for urea. At this 2:1 ratio of urea:methylamine, the potential protein denaturing effects of urea are greatly minimized.

Increases in the levels of cellular osmolytes can be accomplished by different mechanisms (reviewed by Burg 1995). In the kidney, osmolytes like sorbitol and glycerophosphorylcholine are produced by de novo synthesis. Yet others, like betaine and taurine are taken up from the extracellular medium via the action of specific transporters present at the plasma membrane. Within the last few years a number of co-transporters and biosynthetic enzymes responsible for the intracellular accumulation of organic osmolytes have been identified, cloned and characterized (reviewed in Burg 1995). The osmolyte transporters are directly coupled to Na^+ and/or Cl^- uptake enabling the intracellular accumulation of the organic osmolytes using electrochemcial gradients for these ions. Many of these transporters exhibit sequence homology with one another and have a predicted structure of 12 membrane-spanning domains. The promoter regions of the genes encoding these transporters contain sequence-specific elements which appear to be responsible for their higher expression following osmotic shock. It should be emphasized, however, that the accumulation of cellular osmolytes is a relatively slow process, requiring many hours if not days after osmolality is raised. In this regard, it is interesting to note that hypertonicity also results in the activation of the heat shock response. Within 30 min after increasing extracellular NaCl concentration by 100 mM, MDCK cells in culture exhibit a significant induction of mRNA encoding Hsp70 (Cohen et al 1991; Sheikh-Hamad et al 1994). Thus, activation of the heat shock response likely precedes the accumulation of cellular osmolytes and, therefore, may serve to protect the cells until sufficient amounts of the organic osmolytes have accumulated.

MANIPULATING CELLULAR OSMOLYTES FOR CELLULAR PROTECTION AND/OR THE CORRECTION OF FOLDING DEFECTS

The observations that different cellular osmolytes can act to stabilize proteins against proteotoxic conditions, as well as being effective in correcting folding abnormalities associated with specific diseases, have potential ramifications in a number of areas of basic and applied science. First, the ability to manipulate cellular osmolyte levels could lead to new strategies for increasing cellular protection within tissues and organs. Like the situation with the heat shock proteins, increases in the intracellular levels of different organic osmolytes confer added protection to cells upon their exposure to heat shock treatments. For example, addition of either betaine or glycerol to cells in culture causes a marked attenuation of Hsp70 expression following heat shock treatment (Edington et al 1989; Petronini et al 1993; Sheikh-Hamad et al 1994). Moreover, at least in the case of glycerol, addition of the polyol significantly enhanced the survival of cells to heat shock treatments that otherwise would be lethal (Lin et al 1981; Henle et al 1983; Edington et al 1989). Hence, the accumulation of organic osmolytes not only confers added protection to cells confronted with osmotic shocks, but also results in the acquisition of a thermotolerant-like phenotype. Based on these observations we suspect that increases in the levels of cellular osmolytes likely will enhance the protection of cells to a variety of different metabolic insults. Examples with clinical relevance include hemodynamic overload and ischemia/reperfusion injury. Owing to the fact that the genes encoding the different transporters and enzymes responsible for intracellular osmolyte accumulation possess inducible promoter and enhancer elements within their promoter regions, pharmacological ways to increase their expression may eventually be realized.

In the case of already known protein folding abnormalities, for example those associated with particular diseases, the possibility of using cellular osmolytes to correct the folding defect should be considered. As mentioned earlier, many genetically-based diseases are caused by relatively minor mutations that likely result in only subtle protein folding abnormalities. Oftentimes these mutations are not so severe as to inhibit entirely the biological activities of the mutant protein. Eliciting the proper folding and trafficking of these mutant proteins via the use of chemcial chaperones may prove to be an effective strategy for the treatment of these different diseases. Here the success already realized with the ΔF508 CFTR mutant, as well as the prion protein, hopefully will encourage others to examine whether one or more of the different chemical chaperones/cellular osmoytes will correct other protein folding abnormalities. Even if successful, however, the question of how we will increase chemical chaperone levels within the affected tis-sue/organ will require extensive study.

Finally, chemical chaperones may eventually prove useful in the production of commercially/medically important proteins. Novel proteins are expressed in a variety of heterologous systems using recombinant DNA technologies. It is not uncommon for an investigator to experience a problem with the folding and/or secretion of a desired protein when expressed in bacteria, insect cells or different mammalian sources. The manipulation of either the levels of the molecular chaperones, or the concentration of cellular osmolytes could lead to new 'folding environments' that result in the higher expression and/or correct folding of the particular polypeptide of interest. It will be interesting to see whether we can take advantage of the different strategies nature has already designed to deal with the problems a polypeptide encounters in its folding and/or maintainance of biological acitivity within the cell.

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